COMBINATION APPROACH FOR TARGETING MULTIPLE PATHWAYS IN THE MANAGEMENT OF ALZHEIMER'S DISEASE

SUMMARY FOR THE DEGREE OF

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Pharmaceutical Sciences

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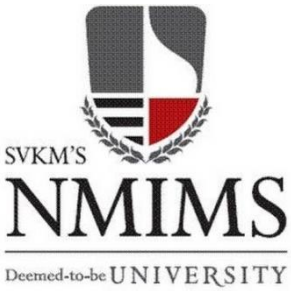

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INTRODUCTION

Alzheimer's disease (AD) is one of the most common types of dementia accounting for about 60% to 80% of dementia cases across globe. It is a chronic and progressive neurodegenerative disorder characterized by cognitive dysfunction and behaviour impairment. Difficulties in remembering recent conversations, names or events are some of the early clinical symptoms; however impaired communication, confusion, behavioral changes, poor judgment, disorientation, difficulty in speaking, swallowing and walking includes late symptoms[1,2].

Prevalence studies of dementia in United States, found that prevalence rates in those aged 65 years and older ranged from 3.6% to 10.7%[3]. The prevalence of dementia in India is 3.7% [4].

Age-specific prevalence of AD and dementia double approximately every 5 years. It is projected that by 2050 the population of 60 years of age shall be almost 2 billion world over. With this increase in the aging population prevalence of AD is going to be soring in coming decades[5]. The researchers with the Institute for Health Metrics and Evaluation at the University of Washington School of Medicine reported at Alzheimer's Association of International Conference (AAIC) 2021 that the number of people with dementia will almost triple to 152 million by 2050 from 57.4 million cases globally in 2019[6]. The recent reports estimate nearly 9.9 million people develop dementia each year; this figure translates into one new case every three seconds. Nearly 60% of people with dementia currently live in low- and middle-income countries and most new cases (71%) are expected to occur in these countries[7].

AD warrant both direct and indirect burden on the healthcare system. The rising cost for the management of AD, impact on household & caregivers is a topic of discussion in recent years[8]. As per World Health Organization (WHO) in 2019, the estimated total global societal cost of dementia was US$ 1.3 trillion, which is anticipated to cross US$ 2.8 trillion by 2030[7,9].

The major impact of burden is expected in low- and middle-income countries due to the lack of social and economic resources to cater to the healthcare needs of rapidly growing aging population[10]. Approximately, 60% of the world's population lives in the Asia Pacific region, with its rapidly aging population; It is expected to have a significant socio-economic burden, the dementia care costs in the region currently stand at US$185 billion[1,11].

AD is a chronic and progressive neurodegenerative disorder characterized by cognitive dysfunction and behaviour impairment[2]. The neurodegenerative process is characterized by an impairment of the synapses with degeneration of the axons and ultimate atrophy of the dendritic

tree and perikaryon[12]. The key pathological hallmark of the AD are noxious extracellular deposits of β-amyloid plaques and intracellular aggregates of hyperphosphorylated tau proteins, neurofibrillary tangles (NFT)[13]. AD is classified as Familial, Early onset Alzheimer's disease (EOAD) and Sporadic AD, Late onset Alzheimer's disease (LOAD). Majority of EOAD are due to mutations in one of the three genes, presenilin-1 (PSEN-1), presenilin-2 (PSEN-2) or amyloid precursor protein (APP)[14]. The Sporadic AD (LOAD) is multifactorial and key risk factors linked are lipid metabolism, oxidative stress, mitochondrial damage, neuro-inflammation and ApoE polymorphism[15]. The data from epidemiological and clinical studies indicates various hypothesis for the pathogenesis of AD; amyloid hypothesis, tau hypothesis, glutamate neurotoxicity/calcium hypothesis, cholinergic hypothesis, mitochondrial cascade hypothesis, metabolic hypothesis and vascular hypothesis[16–18].

In recent times, the understanding of Alzheimer's disease, progression and pathogenesis have been a subject of intense debate and discussion among the scientific community for aiding the development of pharmacotherapy for the management and treatment of the AD. Current treatment options for AD propose two strategies; **1)** symptomatic and **2)** disease modifying approach[19]. At present approved pharmacologic treatment for AD includes cholinesterase inhibitors (ChEIs) and N-methyl-d-aspartate (NMDA) glutamate receptor antagonist [20,21]. Although present pharmacotherapy does not cure or inverse the neurodegeneration of AD, the treatment improves memory loss and cognitive functions. For mild to moderate symptoms, first line of treatment recommended is cholinesterase inhibitors such as donepezil, rivastigmine, galantamine and N-methyl-d-aspartate (NMDA) antagonists and memantine. A combination therapy of donepezil and memantine is also approved by FDA for the treatment of moderate to severe symptoms. But cholinesterase inhibitors have been reported with serious adverse events such as gastrointestinal complaints like nausea, vomiting, loss of appetite, headache, insomnia and dizziness. Further other systemic effects related with cholinergic activity include bradycardia, urinary frequency and syncope. Because of this, close monitoring of the patient and dose titration has been recommended[20,22,23].

Therefore, there is unmet need to develop new therapeutic approach for it is fundamental to pursue new strategies for the development of pharmacotherapy for AD. A variety of natural compounds has been described to have neuroprotective effects which has increased the interest of the researchers and pharmaceutical industry. The inclination of the scientists towards the natural product is increasing day by day not only due to their effectiveness but also due to safety profile. Natural compounds are receiving special attention for the development of

"multiple-targets lead" for the treatment of AD[24]. Flavonoids have demonstrated wide spectrum of health-benefits and are crucial part of various nutraceuticals, pharmaceuticals and cosmetic products[25]. Flavonoids have been attributed to their anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-allergic, anti-thrombotic, anti-viral, hepatoprotective, cardioprotective and neuroprotective properties coupled with their ability to modulate critical cellular enzymes such as lipoxygenase, xanthine oxidase (XO), phosphoinositide 3-kinase and cyclo-oxygenase (COX) [25,26].

Combination of flavonoids with currently approved drug will be a novel approach for the management of AD. The present research work is focused on studying effect of combination of selected flavonoids and FDA approved drug memantine for AD.

RATIONALE

Alzheimer's disease (AD) is a neurodegenerative disease, progressively affecting the memory and cognition. The neuropathology of AD includes aggregation & accumulation of amyloid plaques and neurofibrillary tangles in the brain of AD patients. Neurons are affected in AD patients due to deficit in acetylcholine (ACH) and over activation of NMDA (N-methyl-D-aspartate) receptors. Therefore, increasing the concentration of acetylcholine in brain serves as an important strategy in the development of anti-Alzheimer's drugs. Over activation of NMDA receptors is responsible for neuronal cell apoptosis triggered by glutamate-mediated neurotoxicity. This makes use of NMDA receptor antagonist as one of the major target for the treatment of AD by preventing neurotoxicity[27].

Current treatments available for the management of AD are very limited including acetyl cholinesterase inhibitors like donepezil, rivastigmine and galantamine. All these drugs have limited potential along with side effects such as nausea, bronchoconstriction, hypotension and hepatotoxicity.

Comprehensive research progress on different natural products worldwide shows the remarkable therapeutic effects on CNS. Herbal extracts have rather more remedial benefits and fewer side effects[28]. The knowledge related to the phytochemical, biological and cellular activities of various medicinal plants gives sufficient baseline information for drug discovery and development in treatment of CNS disorders[29]. Flavonoids is one of the important class of phytochemicals with significant effects in neurodegenerative diseases including Alzheimer's disease.

Quercetin and baicalein are important flavonoids with antioxidant, anti-inflammatory, anti-apoptotic, acetylcholinesterase inhibitory activity, neuroprotective action and significant effect in AD.

Further, Memantine, a NMDR antagonist with Acetylcholinesterase inhibitors is FDA approved medication for the management of Alzheimer's disease.

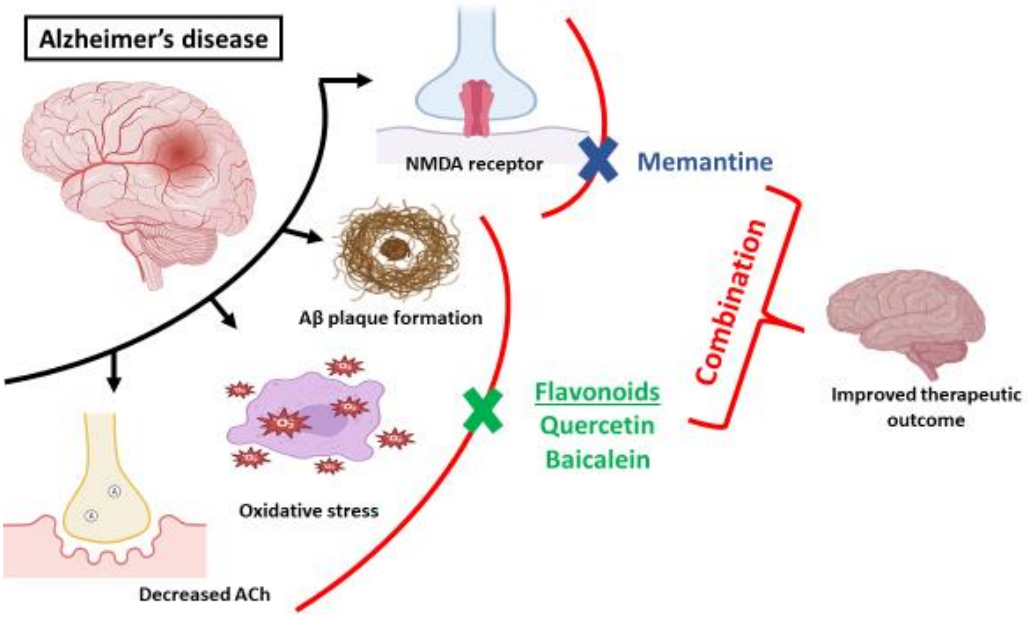

Figure 1: Rationale of the study

Thus, the present work was designed to study effect of quercetin and baicalein alone and in combination with memantine in animal models of Alzheimer's disease.

OBJECTIVES

1. To study the effect of quercetin and baicalein co-administration with memantine in experimental model of $AlCl_3$ induced Alzheimer disease in rats.
2. To study the effect of quercetin and baicalein co-administration with memantine in experimental model of amyloid-β induced Alzheimer disease in rats.

MATERIAL AND METHODS

Drugs and chemicals

Quercetin was procured from Sigma Aldrich (St. Louis, MO, USA). Baicalein was procured from Chemical Center India and Memantine was supplied by Intas as a gift sample. β-Amyloid antibody (mouse), pro BDNF (mouse) antibody and HRP-conjugated secondaryantibodies (anti-rabbit) were purchased from SantaCruz Biotechnology, USA.

Experimental animals

Male albino Wistar rats weighing between 180 and 220 grams were purchased from National Institute of Biosciences, Pune, India. Animals were housed at standard laboratory conditions (22±2 °C, relative humidity of 75±5%, water ad libitum, dark and light cycle for 12 h each) in animal facility. The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) which works under governance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

To study effect of quercetin, baicalein in combination with memantine in $AlCl_3$ induced Alzheimer's' disease.

Induction of diabetes and treatment

- Alzheimer's disease like symptoms were induced in male albino Wistar rats by administration of $AlCl_3$ (100 mg/kg) orally[30]
- $AlCl_3$ solution was freshly prepared in distilled water just before the administration
- Animals were randomized based on their body weight into five groups (n=10)
- 5 % Tween 80 solution was used as vehicle to suspend quercetin, baicalein and memantine.
- Quercetin was administered concomitantly by oral route at doses of 25 and 50 mg/kg, baicalein was administered concomitantly by oral route at doses of 5 and 10 mg/kg, while memantine was administered at dose 20 mg/kg orally, 1 hour after $AlCl_3$ administration daily for 42 days.

Selection of Dose

Selection of dose for quercetin (25 & 50 mg/kg) was based on the previous studies, which showed beneficial effect in the animal model on Alzheimer's disease [31–33]. Selection of dose for the baicalein (5 & 10 mg/kg) was also based on the earlier conducted studies which where baicalein has shown neuroprotective effect in animal models for AD [34–37]. The dose of the memantine (20 mg/kg) has been selected based on various published literature. In many previous animal studies, the dose of the memantine selected was ranged from 5-30 mg/kg/day. However, in clinical practice, it has been observed that a stable dose of 20 mg/day memantine in patients with Alzheimer's disease (AD) produces a steady-state plasma drug level of approximately 0.5 μmol/L[38,39]. Further, it has also been reported that in male rats (200–300 g) sub-chronic administration of 20 mg/kg per day is of therapeutic relevance as this dose produces plasma levels similar to those mediating clinical effects in the treatment of demented patients. This dose also leads to brain levels sufficient to affect NMDA receptors. The higher dose is likely to be selective at NMDA receptors but side effects are expected [40,41]. Hence in the present study we have used dose of 20 mg/kg/day.

Grouping of animals for quercetin and quercetin with and memantine $AlCl_3$ induced AD study

Effect of quercetin and quercetin with and memantine in aluminium chloride ($AlCl_3$) induce Alzheimer's disease in rats	
Group I	Normal Control (Distilled water *p.o.*)
Group II	Disease Control ($AlCl_3$-100 mg/kg *p.o.*)
Group III	$AlCl_3$-100 mg/kg+memantine hydrochloride (20 mg/kg *p.o.*)
Group IV	$AlCl_3$ (100 mg/kg) + quercetin (50 mg/kg *p.o.*)
Group V	$AlCl_3$ (100 mg/kg) + [memantine hydrochloride (20 mg/kg *p.o.*)+ quercetin (25 mg/kg *p.o.*)]
Group VI	$AlCl_3$ (100 mg/kg) + [memantine hydrochloride (20 mg/kg *p.o.*)+

	quercetin (50 mg/kg *p.o.*)]

Grouping of animals for baicalein and memantine $AlCl_3$ induced AD study

Effect of baicalein in aluminium chloride ($AlCl_3$) induced Alzheimer's disease in rats	
Group I	Normal Control (Distilled water *p.o.*)
Group II	Disease Control ($AlCl_3$ - 100 mg/kg *p.o.*)
Group III	$AlCl_3$- 100 mg/kg + memantine hydrochloride (20 mg/kg *p.o.*
Group IV	$AlCl_3$ - 100 mg/kg + baicalein (10 mg/kg *p.o.*)
Group V	$AlCl_3$ - 100 mg/kg + [memantine hydrochloride (20 mg/kg *p.o.*)+baicalein (5 mg/kg *p.o.*)]
Group VI	$AlCl_3$ - 100 mg/kg + [memantine hydrochloride (20 mg/kg *p.o.*)+baicalein (10 mg/kg *p.o.*)]

Behavioral Assessment

I. Locomotor activity

Each animal was kept in actophotometer and locomotor activity was observed for the period of five minutes on day 21 & 42. The actophotometer consisted with infrared photocells. As thelight beam gets cut off due to the crossing of animal, it was considered as one count. Readingswere expressed in terms of counts/5 minutes[30].

II.Morris water maze (MWM)

MWM test is usually performed to assess spatial memory in rodents. A large circular swimming tank consisted of dimensions like 150 cm diameter and 45 cm was marked into four equal quadrants and filled with water up to suitable height. In acquisition trial, the submerged platform with dimension (10 cm X 10 cm), was kept in water 1 cm above the surface. The animal was

placed in one of the quadrants of swimming tank and allowed for 120 seconds to find the platform and guided if failed and then placed up to 30 seconds on the platform. For each animal, daily four trials were given for four consecutive days (17^{th} to 20^{th} day) as a part of training. In retention trial, by using milk powder water made opaque to hide the submerged platform and kept 1 cm below the water surface. On day 21 and 42 each animal was placed in the tank similarly as acquisition trial and retention of memory of the animal was determined. Escape latencies of animals were measured by calculating the time taken to find the hidden platform by the rodents in tank[30,42].

III.Elevated plus maze (EPM)

The EPM is a test measuring anxiety like behaviour in rodents. The apparatus consisted of two open arms with the dimension (50 cm x 10 cm) which are transverse with two closed arms with high walls in similar dimensions. Both the arms of EPM were connected in plus shape design with central square (10 cm x 10 cm) and maintained elevated height up to 50 cm abovethe ground level. The apparatus was designed in such manner that similar arms are opposite toeach other. During the study, exploration time was kept 5 minutes for each animal in maze. In acquisition trial, the animal was placed at one end of open arm and the time required to move from open arm to closed arm of animal was recorded as initial transfer latency (ITL). and then after training animal was returned to the home cage. After 24 hours of ITL, each animal was again placed in an open arm of maze and the retention of memory was evaluated and measured as 1^{st} transfer latency on day 21 and 2^{nd} transfer latency on day 42[30,43].

IV.Passive avoidance test (PA)

PA test is a fear-aggravated test used to assess learning and memory in rodents. The apparatus consisted of light and dark compartment with dimensions of each (25 x 25 x 25 cm). The light compartment was illuminated with a 40W bulb and a dark chamber, separated by a guillotine door. The dark compartment equipped with a metal grid floor at the bottom with a shock generator to induce electric stimuli. During the acquisition trial, the animal was placed inside the light compartment for the period of 60 seconds for habituation. Then guillotine door was opened and time taken by animal to move in dark compartment was noted as pre-shock latency. After that in the dark compartment, a mild electric stimulus of 0.5 mA was given to animal for2 seconds. The animal was removed from the chamber and returned back to cage. Retention trial was performed after the 24 hours of acquisition trial, in which time taken by animal to step from light compartment to dark compartment was noted as post-shock latency during this acquisition

trial electric stimulus was not given in the dark compartment once animal entered[43].

Assessment of brain oxidative stress markers

Collection of brain & preparation of brain homogenate

At the end of behavioural assessment, all the animals were sacrificed by CO_2 asphyxiation. Brain was collected out and cortex and hippocampus part of the brain were isolated andstored at -80ºC were used for preparation of brain homogenate. Hippocampus and cortex were homogenized in 10 volumes of ice-cold 0.1M phosphate buffer solution (pH 7.4) by usingprobe homogenizer. The tubes were immersed in ice to avoid rise in temperature. Different fractions of the brain homogenates were prepared as per following method.

Total homogenate was centrifuged at 2500 g for 20 min at 4˚C to prepare post nuclear supernatant (PNS). Total homogenate was centrifuged at 10000 g for 20 min at 4 ˚C to prepare post mitochondrial supernatant (PMS).

- Determination of brain protein content by Lowry's method

The protein content of the brain homogenate was measured according to the Lowry's method 1951[44].

Assessment of oxidative stress parameters

The level of glutathione in the hippocampus and cortex was evaluated as per the method described by Ellman, 1959[45]. Total protein content was determined as per method described by Lowry et al., 1951[44]. The catalase activity was determined as per the method by Lück et al 1965[46] in post-nuclear fraction of homogenate. The super oxide dismutase (SOD) activity was evaluated by the method according to Paoletti eta al 1986 & 1990[47,48] in post-mitochondrial fraction. MDA level was determined in brain homogenate by using the method described by Ohkawa et al., 1979[49].

Acetylcholinesterase activity (AChE)

The acetylcholinesterase activity in brain homogenate was estimated as per the method described by Ellman et al., 1961[50].

Histopathological studies

Brains were stored in 10% neutral buffered formalin (NBF) (4 g of sodium phosphate monobasic anhydrous, 8.15 g sodium phosphate dibasic dihydrate, 100 ml formalin, and 900 ml distilled water) were used for histopathology study. Formalin fixed brain were trimmed

longitudinally and routinely processed. Brain processing was done to dehydrate in ascending grades of alcohol, clearing in xylene and embedded in paraffin wax. It was fixed inparaffin blocks and thin transverse sections of 5 µm thickness were taken using microtome (Leica, USA) of hippocampus and cortex region. The sections were stained with hematoxylin and eosin (H&E) and Congo red dye and examined under digital microscope (Motic, Canada).The observations of the sections were carried out at 400X. For histology analysis three slides per group were evaluated for cortical and hippocampal region (complete section). Morphometric analysis was carried out for evaluation of the extent of neurodegeneration & amyloid-β deposition in brain and severity index was considered as No Abnormality Detected, Minimal (<1%), Mild (1-25%), Moderate (26-50%), Marked/Moderately Severe (51-75%), Severe (76-100%) and distribution was recorded as focal, multifocal and diffuse[51,52].

Immunohistochemistry (IHC) for determination of amyloid-β & BDNF expression

Amyloid-β and BDNF expression in hippocampus & cortex were carried out by immunohistochemical staining as per earlier method described by Dugich-Djordjevic et al., 1995 and Rajamohamed sait and Sigurdsson, 2012[51,53]. Paraffin wax embedded tissue blocks were cut and transverse sections at 5-6 µm thickness were taken with the microtome(Leica, USA) and placed on slides coated with Poly-L-Lysine and placed in an incubatorovernight at 37°C for 1 hour. Further these sections were deparaffinized, rehydrated andincubated with citrate buffer, pH 6 at decloaking chamber. Slides were incubated in 3% hydrogen peroxide block for 20 minutes to block endogenous peroxidase. Human anti-rat β-Amyloid (b-4) antibody and Rabbit anti-BDNF antibody (pro-BDNF: 5H8) (Santa Cruz Biotechnology, Inc., USA) were applied as the primary antibody and secondary antibody like peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., USA) used. The staining was visualized by reaction with diaminobenzidine (DAB) reagent and further counter-stained with hematoxylin. The sections were rinsed with Tris buffer saline (TBS) and dehydrated in alcohol and cleared in xylene prior to mounting using DPX. All the sections were examined under the digital microscope (Motic, Canada) to record the intensity of antigen antibody reaction. The observation of the sections was carried out at 400X. Forimmunohistochemistry analysis three slides per group were evaluated for hippocampal & cortical region (complete section). Morphometric analysis was carried to observe extent of immunoreactivity.

To study effect of quercetin, baicalein in combination with memantine in Amyloid-β induced Alzheimer's disease

Amyloid-β induced Alzheimer's disease

Preparation of $A\beta_{1-42}$ protein solution:

$A\beta_{1-42}$ protein fragment was dissolved in sterile double-distilled water, to make the concentration of 4µM. This solution was incubated at 37^{o}C for 4 days prior to use.

Neurosurgery procedure

- Animals were anesthetized by using Ketamine (80 mg/kg body weight) and Xylazine (20 mg/kg body weight) injection.
- Alzheimer's disease in albino Wistar rats was induced by intracerebroventricular (i.c.v.) injection of amyloid-β protein (Aβ1-42) at concentration of 4µM/rat by using stereotaxic apparatus, 20 days prior to treatment.
- The skin of animals was shaved, disinfected and cut with a sterile scalpel to expose the skull. Stereotaxic co-ordinates of rat brain were decided relative to bregma: AP= -0.8 mm, ML= +1.2 mm, DV= -3.6 mm.
- The injection volume was kept 4 µl and delivered gradually (1 µl/min). 0.9% normal saline solution was delivered to the sham control animals.
- The animals were given post-operative care comprising of gentamicin (5mg/kg; s.c.) for 7 days along with Neosporin powder sprinkling and betadine application on suture sites.
- Treatment groups received quercetin at dose 50 mg/kg and baicalein at dose 10 mg/kg orally for 21 continuous days.
- Selection of dose for each drug was based on reported studies and literature.

Grouping of animals for quercetin and quercetin with memantine Amyloid-β induced AD

Effect of quercetin in amyloid-β (Aβ $_{1-42}$) induced Alzheimer's disease in rats	
Group I	Normal Control - (0.9% saline *i.c.v.*)
Group II	Disease Control - $A\beta_{1-42}$ [4 µM (4 µl i.c.v.)]
Group III	$A\beta_{1-42}$ [4 µM (4 µl *i.c.v.*)] + memantine hydrochloride (20 mg/kg *p.o.*)
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Group V	$A\beta_{1-42}$ [4 µM (4 µl *i.c.v.*)] + [memantine hydrochloride (20 mg/kg *p.o.*)+ quercetin (25 mg/kg *p.o.*)]
Group VI	$A\beta_{1-42}$ [4 µM (4 µl *i.c.v.*)] + [memantine hydrochloride (20 mg/kg *p.o.*)+ quercetin (50 mg/kg *p.o.*)]

Grouping of animals for baicalein and baicalein with memantine in Amyloid-β induced AD

Effect of baicalein in amyloid-β (Aβ $_{1-42}$) induced Alzheimer's disease in rats	
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A. Assessment of oxidative stress markers

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Immunohistochemistry for determination of amyloid-β & BDNF expression

Amyloid-β and BDNF expression in hippocampus & cortex were carried out by immunohistochemical staining as per earlier method described by Dugich-Djordjevic et al., 1995 and Rajamohamed sait and Sigurdsson, 2012[51,53]. Paraffin wax embedded brain blocks were cut and transverse sections at 5-6 µm thickness were taken with the microtome (Leica, USA) and placed on slides coated with Poly-L-Lysine and placed in an incubator overnight at 37°C for 1 hour. Further these sections were deparaffinized, rehydrated and

incubated with citrate buffer, pH 6 at decloaking chamber. Slides were incubated in 3% hydrogen peroxide block for 20 minutes to block endogenous peroxidase. Human anti-rat β-Amyloid (b-4) antibody and Rabbit anti-BDNF antibody (pro-BDNF: 5H8) (Santa Cruz Biotechnology, Inc., USA) were applied as the primary antibody and secondary antibody like peroxidase-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., USA) used. The staining was visualized by reaction with diaminobenzidine (DAB) reagent and further counter-stained with hematoxylin. The sections were rinsed with Tris buffer saline (TBS) and dehydrated in alcohol and cleared in xylene prior to mounting using DPX. All the sections were examined under the digital microscope (Motic, Canada) to record the intensity of antigen antibody reaction. The observation of the sections was carried out at 400X. For immunohistochemistry analysis three slides per group were evaluated for hippocampal & corticalregion (complete section). Morphometric analysis was carried to observe extent of immunoreactivity.

Statistical analysis

In efficacy study, the differences among treatment and control groups were analyzed by using GraphPad Prism V 5.0 for Windows. Behavioural parameters were assessed by two-way ANOVA (analysis of variance) followed by *Bonferroni's test* and the significance level were determined within and between groups. Oxidative stress parameters, AChE activity and immunohistochemistry quantification data were assessed by one-way ANOVA followed by Dunnett's multiple comparison tests. All data are expressed as mean ± standard error of mean (SEM); *p* value less than 0.05 was considered to be significant. Optical density data of ICH images was also evaluated using ImageJ 1.8.0 software.

To study effect of quercetin and quercetin in combination with memantine in $AlCl_3$ induced Alzheimer's disease

The study was performed to investigate the effect of quercetin with memantine against the protection of $AlCl_3$-induced oxidative stress, memory degradation, and neuronal cell death. It has been reported that the administration of $AlCl_3$ is responsible for oxidative stress, which leads to the peroxidation of lipid membranes and cell apoptosis. Further, the dysfunction of neuronal cells and progressive death of neurons are also associated with oxidative stress[33,54].

From the study result, it can be concluded that the administration of quercetin with memantine has significantly improved behavior parameters, learning, and spatial memory. The administration of quercetin with memantine has shown significant improvement in GSH, catalase, and SOD levels and a decrease in MDA levels. It is an indication of a reduction in the oxidative stress parameters, which are critical to neurodegeneration or mitochondrial damage. The ability of quercetin to inhibit oxidative stress comes from the property of quenching reactive oxygen species. Quercetin has also shown significant acetylcholinesterase inhibitory activity. Inhibition of amyloid-β plaque formation and increase in the levels of BDNF in the brain tissues of animals treated with quercetin and memantine signaling synergistic treatment effect.

The study results indicated that quercetin with memantine protected the degradation of learning and loss of memory.

To conclude, the data from our study directed that quercetin has multidimensional therapeutic effects such as antioxidant, anti-inflammatory, and acetylcholinesterase inhibiting activity. The present study results indicated different pathways for both drugs, and their synergistic activity was also observed. However, to confirm the clinical benefits of the quercetin and memantine combination, more studies are required to be performed.

To study effect of baicalein and baicalein in combination with memantine in $AlCl_3$ induced Alzheimer's disease.

The study result revealed reduced locomotor activity and forelimb grip strength, demonstrating a compromised neuronal and or muscular function in β-Amyloid Precursor Protein-Deficient Mice has been reported [55]. In present study, significant impairment in the locomotor activity was observed in disease control. Treatment of baicalein with memantine at a dose of 20+10 mg/kg improved the locomotor activity in experimental animals.

Morris water maze test was used to assess learning and acquisition of spatial memory [56]. In present study, an increase in escape latency in the treatment group of baicalein with memantine at a dose of 20+10 mg/kg suggested recovery of neurotoxicity induced by $AlCl_3$ in brain.

EPM test measures transfer latency which helps to understand spatial memory and learning. Chronic $AlCl_3$ exposure decreases the open arm exploration and increases the transfer latency expressing the anxiogenic property [57]. The study results showed, treatment of baicalein with memantine at a dose of 20+10 mg/kg improvised the transfer latency. Passive avoidance test is useful tool for assessment of learning, memory and cognition. Decrease in post shock latency time by $AlCl_3$ administration was an indication of progressive memory impairments [58]. The results of the present study indicated increase in post shock latency in the treatment group of baicalein with memantine at 20+10 mg/kg dose.

Treatment with $AlCl_3$ 100 mg/kg orally for 42 days has ensured significant increase in oxidative stress markers, resulting in neurodegeneration through lipid peroxidation. MDA is one such oxidative stress marker which leads to reduced axonal mitochondrial turnover and disruption of Golgi [59]. The results indicated significant depletion of lipid peroxidation in hippocampus and cortex by treatment of baicalein with memantine. SOD is considered as a frontline defense mechanism, plays critical role in catalyzing highly reactive free radical (O_2-) into hydrogen peroxide (H_2O_2) and molecular oxygen [60]. Prominent rise in SOD levels as compared to disease control group were observed in hippocampal and cortex region in the treatment group of baicalein with memantine (20+10 mg/kg). Catalase is another prime antioxidant enzyme involved in conversion of hydrogen peroxide into water and oxygen. The results showed decrease in catalase activity in $AlCl_3$ treated disease control group which is in line with published studies [61]. Treatment of baicalein with memantine (20+10 mg/kg) group showed improvement in catalase levels in both hippocampus and cortex. GSH system is

required for cellular detoxification of reactive oxygen species within the cells [62]. Baicalein with memantine treatment significantly increased the cellular GSH levels. Thus, an improvement in levels of antioxidant enzyme system when treated with baicalein and memantine revealed its strong antioxidant activity in $AlCl_3$ induced neurotoxicity.

Acetylcholine (ACh) is a neurotransmitter involved in both central and peripheral nervous system supporting cognitive and physiological functions within the body. Acetylcholinesterase (AChE) is cholinergic enzyme involved in termination of neuronal transmission and signalling between the synapses. Alteration in AChE activity is sign of learning and cognitive disability. The present study result indicated that treatment of baicalein with memantine at a dose of 20+10 mg/kg significantly reduced the AChE activity in hippocampus and cortex region when compared with disease control group which supports its use in the management of AD.

The β-Amyloid plaques are hallmark in the pathogenesis of AD. Excessive deposition of Aβ induces neuronal dysfunction and synaptic coordination. Administration of $AlCl_3$ for 42 days in experimental animals enhanced an expression of amyloid-β in hippocampus and cortex. Treatment of baicalein with memantine has shown reversing of β-Amyloid plaque formation induced due to the administration of $AlCl_3$.

Expression of BDNF is key factor in determination of neuronal health. In present study, BDNF deficiency was evident in $AlCl_3$ treated group. Treatment of baicalein with memantine enhanced the expression of BDNF in hippocampus and cortex.

To study effect of quercetin and quercetin in combination with memantine in Amyloid-β induced Alzheimer's disease.

The study result indicated significant impairment in the locomotor activity of the disease control animals. Animal group treated with quercetin and memantine at a dose of 20+50 mg/kg has shown improvement in the locomotor activity when compared with the disease control group.

The result form the MWM indicated that the memory and learning skills of animals were improved in animals treated with quercetin and memantine at a dose of 20+50 mg/kg. The smaller escape latency in the treatment group of quercetin and memantine is an indication of the improvement in disease state as compared to the diseases control group.

In EPM test, reduction in the transfer latency has been observed in the disease control group when compared with sham operated animals. In contrast quercetin with memantine at a dose of 20+50 mg/kg facilitated consolidation of memory confirmed by increase in the transfer latency as compared to the latency of the animals from the disease control group.

In passive avoidance, the memory retention was evaluated by measuring pre and post shock latency. The disease control animals have shown decline in the latency when compared with normal control group. On the other hand, significant increase in the post shock latency has been observed in the treatment group of quercetin with memantine at a dose of 20+50 mg/kg as compared to the disease control group as well as treatment group of memantine.

The literature suggest that Aβ plaque have detrimental effect on the mitochondrial redox activity and is also liable for peroxidation of membrane lipids resulting in cell apoptosis [63,64]. An increase in the production of oxidative free radicals engulfs the capability of antioxidants to neutralize them, leading to increased call damage [65]. In this study, we evaluated various oxidative stress enzymes and the result from the study indicated quercetin with memantine diminished the cytotoxic effect of Aβ by ameliorating antioxidant effect and suppression of lipid peroxidation. Further the results also demonstrated free radical scavenging effect of quercetin with memantine at a dose of 20+50 mg/kg [66,67].

Acetylcholinesterase inhibitors (AChEIs) re-establish the normal function of cholinergic neurons for synaptic transmission and hence are prescribed for the management of AD [68]. However, serious adverse events such as nausea, vomiting and diarrhea have been attributed to the currently available AChE inhibitors. Therefore, a natural product with an

acetylcholinesterase inhibitory activity thought to be an appropriate alternative. Various in-vitro studies have been demonstrated AChE (IC50=353.86 μmol/L) and BChE (420.76 μmol/L) inhibitory activity for quercetin [69]. The present study result indicated increase in AChE activity in Aβ treated group; on the contrary, significant inhibitory effect was seen in the animals administered with quercetin and memantine.

Microscopic examination of H&E stained brain tissues from normal control group revealed no lesion of pathological mutilation. The disease control group showed multifocal moderate neuronal degeneration at hippocampus and cortex. However, animals treated by quercetin with memantine at a dose 20+50 mg/kg showed focal minimal neuronal degeneration at hippocampus and cortex, which demonstrated that treatment with quercetin and memantine have reversed the neurodegeneration effect of Aβ.

In the Congo red stain section of brain revealed significant extent of deposition of Aβ plaque. The multifocal moderate Aβ deposition in both hippocampus and cortex was seen in animals from the disease control group, which was absent in the normal control group. However, animals treated with quercetin and memantine at 25+50 mg/kg showed focal minimal to mild deposition of Aβ in both hippocampus and cortex, which indicted that combination of quercetin and memantine could inhibit the formation of Aβ plaque in the brain.

In immunohistochemical (IHC) studies, microscopic examination of brain sections from animals of normal control group did not show any signs of Aβ deposition in both hippocampus and cortex. But disease control group showed moderately enhanced expression of Aβ plaques in both hippocampus and cortex. However, treatment by quercetin with memantine at a dose of 20+50 mg/kg showed mild enhanced expression of Aβ in cortex and hippocampus. Thus, it indicated that treatment of quercetin with memantine at a dose of 20+50 mg/kg has been effective in inhibiting the formation of Aβ.

BDNF has neuroprotective effect, acts by inhibiting inflammatory factors leading to decreasing apoptosis. BDNF functions through tropomycin receptor kinase B (TrkB) and p75 neurotrophin (p75NTR). Through TrkB receptor, which is considered as essential for the activation of BDNF, mediates various intracellular signaling pathways, like PI3K/Akt, which has a critical role in synaptic plasticity and neuronal survival [70–72]. We studied an expression of BDNF by microscopic examination through immunohistochemical stain. The results from the examination indicated decrease in expression of BDNF in hippocampus and cortex of disease control group as compared to the normal control group. However, treatment of

quercetin with memantine at a dose of 20+50 mg/kg indicated moderately enhanced expression of BDNF in hippocampus and cortex. The optical density data of the IHC studies also supports the microscopic examination observations.

To study effect of baicalein and baicalein in combination with memantine in Amyloid-β induced Alzheimer's disease.

The study results indicated significant impairment in the locomotor activity of the disease control animals. However, treatment of baicalein with memantine at a dose of 20+10 mg/kg has shown substantial improvement in the locomotor activity when compared with the disease control group. In the MWM, the animals treated with baicalein and memantine at a dose of 20+10 mg/kg indicated extensive improvement in the memory and learning skills as compared to the diseases control group. Further, reduction in the transfer latency of disease control group and substantial increase in the animals treated with baicalein and memantine at a dose of 20+10 mg/kg was observed in EPM. Moreover, In PA test decline in the memory retention was observed in disease control group. On the contrary significant increase in the memory retention was observed in the treatment group of baicalein with memantine at a dose of 20+10 mg/kg. The study results from behavioural assessment indicated that memory, learning and cognitive characteristics in the animals injected with $A\beta_{(1-42)}$were declined over a period of the study whereas the improvement was observed in the treatment group of baicalein and memantine.

Furthermore, the animal model of AD by injecting Aβ through intracerebroventricular (i.c.v.) injection for pathophysiological traits of AD is more relevant to the sporadic AD, clinical symptoms such as oxidative stress; neuroinflammation and cholinergic dysfunction were also witnessed in this animal model [73]. This allowed us to understand the effect of treatment of baicalein with memantine in the management of AD.

We evaluated various oxidative stress markers like MDA, SOD, Catalase and GSH. The study results indicated antioxidant properties of baicalein with memantine at a dose of 20+10 mg/kg.

The objective of the AD treatment is to re-establishing the cholinergic functions which were disturbed due to the dysfunction of synapses leading to memory impairment and cognitive abnormalities [74]. The study results indicated that treatment of baicalein with memantine at a dose of 20+10 mg/kg significantly reduced the AChE activity in hippocampus and cortex region which supports an improvement in the memory and cognition.

The results from the behavioural, memory and learning models also establishes the efficacy of the baicalein and memantine combination therapy against oxidative and neuronal damaged induced by Aβ.

The histopathological evaluation of H&E and Congo Red stain was performed to assess the extent of neurodegeneration and Aβ plaque formation in the present study [75,76]. Histopathological studies in the normal control group by H&E stained brain tissue revealed no lesions of pathological significance. On the contrary, disease control group showed multifocal moderate neurodegeneration in hippocampus and cortex tissues. However, animals treated by baicalein with memantine at a dose 20+10 mg/kg showed focal minimal neuronal degeneration in hippocampus and cortex, which establishes the role of combination treatment of baicalein and memantine in the reversal of neurodegeneration caused by Aβ. Further, the Congo red stained sections of the brain revealed significant extent of Aβ plaque deposition in hippocampus and cortex in animals from the disease control group. However, animals treated with baicalein and memantine at 25+10 mg/kg showed focal minimal to mild deposition of Aβ in both hippocampus and cortex, which indicated anti-amyloid plaque activity of combination therapy of baicalein with memantine.

Immunohistochemical (IHC) evaluations have also confirmed the reversal of β-Amyloid plaque in the animal group treated with baicalein and memantine at a dose of 20+10 mg/kg. Further, expression of BDNF which is an indicator of neuronal health, the IHC evaluation of disease control group showed BDNF deficiency. However, the evaluation from the baicalein with memantine treated group have shown enhancement in the expression of BDNF in hippocampus and cortex.

CONCLUSION

The results from the studies indicated that combination treatments of quercetin and baicalein with memantine have demonstrated synergistic effect in $AlCl_3$ and Amyloid-β induced AD.

Thus, it can be concluded that treatment of quercetin and baicalein with memantine could be an important approach for the management of AD.

PUBLICATIONS FROM PhD Work

Publications from PhD

Sr No	Details	Journal	Impact Factor	Status
1.	Neuroprotective effect of quercetin and memantine against AlCL3induced neurotoxicity in albino Wistar rats	Molecules	4.927	Accepted
2.	Effect of Baicalein with Memantine in Aluminium Chloride Induced Neurotoxicity in Wistar Rats	Frontiers in Pharmacology	5.988	Revision submitted
3.	Cognitive enhancement and neuroprotective effect of Baicalein with Memantine in Amyloid-β Induced Alzheimer's disease in rat model	Antioxidants	7.675	Under Review
4.	Neuroprotective effect of quercetin administered with memantine against Beta-Amyloid induced Alzheimer's disease in Rats	Pharmacology Biochemistry & Behaviour	3.697	Ready for Submission

Presentations

1. **Ratnakar P Jadhav**, Yogesh A Kulkarni. Effect of combination of Baicalein and Memantine in animal model of Alzheimer's Disease. ***9th International Congress of the Society for Ethnopharmacology, India. JSS College of Pharmacy***, JSS Academy of Higher Education & Research Mysuru, India. April 22-24, 2022

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